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TESTING OF THE BIO-SEEQ® (SMITHS DETECTION HANDHELD PCR INSTRUMENT): SENSITIVITY, SPECIFICITY, AND EFFECT OF INTERFERENTS ON *FRANCISELLA TULARENSIS* ASSAY PERFORMANCE

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14. ABSTRACT
Smiths Detection-Edgewood (SDE), Inc., recently developed a handheld PCR instrument, the Bio-Seeq®, an updated, redesigned version of the small, portable PCR instrument previously known as the Handheld Advanced Nucleic Acid Analyzer (HANAA). The SDE developed the Bio-Seeq® to provide a portable platform for first responders to use for detecting biological threats in civilian areas. To be ready for introduction into the marketplace, the instrument must be accompanied by a menu of reagents that will enable the user to detect the presence of pathogens in environmental samples. In previous work, SDE obtained probe and primer sequences constituting an assay for a gene present in *Francisella tularensis,* the causative agent of tularemia. The SDE incorporated this probe and primer set into dried reagent beads, which also contain reagents required for an internal control. These, in turn, are part of a self-contained sampling device that contains buffer and the PCR reagent beads. This report details an analysis of the assay for *F. tularensis*, including the sensitivity and specificity of the assay, and the effect of some common nontarget (interferent) materials on the performance of the assay.

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PREFACE

The work described in this report was authorized under the Cooperative Research and Development Agreement, Project No. 0309C, between the U.S. Army Edgewood Chemical Biological Center and Smiths Detection-Edgewood. This work was started in December 2003 and was completed in January 2004.

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1. INTRODUCTION AND BACKGROUND

In May 2003, the U.S. Army Edgewood Chemical Biological Center (ECBC) and Smiths Detection-Edgewood (SDE), Inc. entered into a Cooperative Research and Development Agreement (CRADA), Project 0309C, for the purpose of developing assays for SDE's handheld PCR instrument, the Bio-Seeq®. The SDE developed the Bio-Seeq® to provide a portable platform for use by first responders to detect biological threats in civilian areas. The Bio-Seeq® is an updated, redesigned version of a small, portable PCR instrument previously known as the Handheld Advanced Nucleic Acid Analyzer (HANAA).[1,2] At least one reference to its use in detecting a BW agent exists in the peer-reviewed literature.[3] Information on the Bio-Seeq® is available from the manufacturer at http://63.89.158.169/products/Default.asp?ProductID=6.

To be ready for introduction into the marketplace, the instrument must be accompanied by a menu of reagents that will enable the user to detect the presence of pathogens in environmental samples. Assays that are designed for any instrument that can accommodate real-time fluorogenic PCR can most likely be adapted for use with the Bio-Seeq®. Molecular biologists at ECBC have experience in the development of real-time fluorogenic PCR assays (TaqMan) for pathogen detection.

The SDE has formulated probe and primer sequences for detecting *Francisella tularensis*, the causative agent of tularemia.[4] The primers and probe have been formulated into dried reagent beads, which also contain reagents required for an internal control. These in turn are part of a self-contained sampling device that contains buffer and the PCR reagent beads.

2. OBJECTIVE

The objective of the work reported here was to answer the following questions:

a. Is the assay (the probe and primer set) specific for *Francisella tularensis*?

b. How sensitive is the assay when target cells or spores are applied directly to the consumable sampler?

c. What is the effect of some common nontarget (interferent) materials on the performance of the assay when target cells or spores are used?

3. SCOPE

This study is the second part of an overall plan to test the sensitivity, specificity, and resistance to interferents of three assays designed for use in the Bio-Seeq® instrument. The probe and primer sets in the assays are designed to detect one of the following threat agents: *Bacillus anthracis*, *Francisella tularensis*, and *Yersinia pestis*.

4. METHODS AND MATERIALS

4.1 Instrument Description.

Smiths Detection-Edgewood, Inc. is the manufacturer of the Bio-Seeq® instrument (Figure 1). Two Bio-Seeq® units (s/n 115, and another unit dubbed thermal paste) were provided to ECBC by SDE. Each unit contains six thermocycler modules that are independently programmable and operable. Each module can be run separately. The instrument has an LCD panel that allows an operator to enter, store, and run programs that instruct each module to heat and cool a PCR reaction tube to a specified temperature for a specified length of time, within the operating parameters of the instrument. A user can also monitor and gather data from each module independently using a PC that is running proprietary software developed by SDE (latest version at this writing is 1.21). When connected to a PC, the instrument will graphically display the development of the fluorescent signal generated by a PCR assay reaction in real time, as well as the signal generated by internal positive control reagents, which are also contained in the reagent beads.

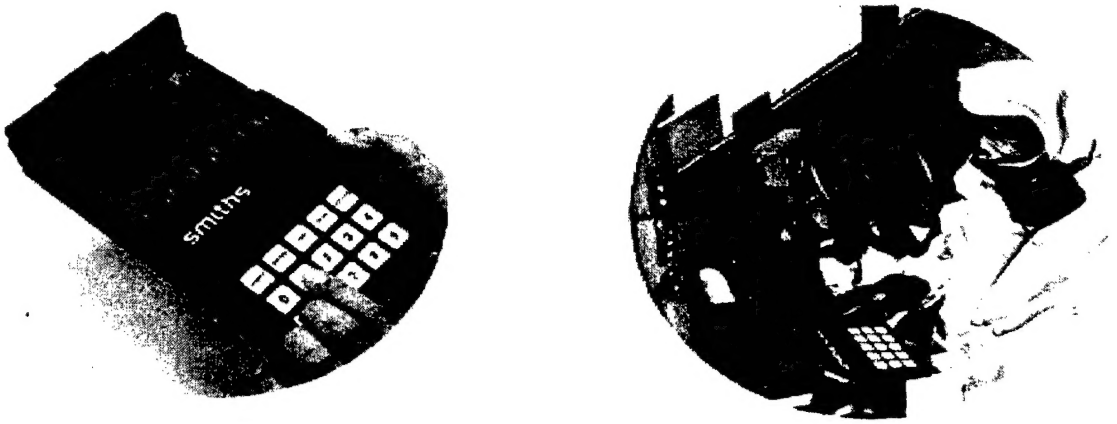

Figure 1. Bio-Seeq® Instrument. Left: the lightshield is open, revealing the openings for 6 Bio-Seeq® tubes. Right: an illustration of the concept of operation. An operator, wearing protective equipment in a potentially hazardous environment, is about to conduct an assay.

The operator conducts an assay using a consumable sampling and reaction tube assembly (a consumable, Figure 2). The consumable is supplied in two assembled pieces. One piece consists of a buffer cup housing with a small handle that contains the buffer cup. Buffer is contained in the cup by a thin breakable plastic film. The second piece consists of a housing called a reagent base, which contains a hollow plunger tipped with a porous swab. Within the hollow plunger are three dried beads, which contain the assay reagents, and an inert mixing bead. A hole at the end of this piece leads into the attached clear plastic reaction tube.

To conduct an assay, the operator removes the two pieces from their packaging, and applies the swab-tipped end of the reagent base to the surface to be sampled. The swab tip is then inserted into the open end of the buffer cup holder and twisted. Threads on the buffer cup housing and the reagent base cause the swab tip to press against and break the film containing the buffer, which carries sample material through the porous tip and into the interior of the hollow plunger. By allowing the buffer to reside in the plunger for a 90-s period, the buffer dissolves the reagent beads. The operator then shakes the device for 20 s causing the mixing bead to mix the sample, the buffer, and the assay reagents. The operator "whips" the assembled consumable to draw the aqueous reaction mixture into the reaction tube, which is then inserted into one of the six PCR modules in the Bio-Seeq® instrument. The operator then starts the program of PCR temperatures and durations appropriate to the particular assay reagents for that test.

4.2 Bacterial Strains, Culture Methods, and DNA Isolation.

The strains used in this study are listed in Table 1. All strains listed (except *F. tularensis)* were obtained from an in-house culture collection at ECBC; many are available from ATCC (Manassas, VA) or the Bacillus Genetic Stock Center (Ohio State University, Columbus, OH). All strains used in this study were non-pathogenic, and were handled according to BSL-2 practices. DNA from several strains of *F. tularensis* was obtained from an in-house collection of bacterial genomic DNA samples. Other, non-pathogenic strains of *F. tularensis* were grown for DNA isolation and cell isolation on solid cysteine heart agar supplemented with hemoglobin solution (Difco), prepared according to the label instructions. Petri dishes containing *F. tularensis* were grown for 4-7 days at 37°C in a static incubator. Cells were harvested by flooding the petri dishes with 10 mM Tris HCl buffer, pH 7.4 (Sigma Company, St. Louis, MO), followed by centrifugation. DNA was extracted from bacterial cells with QIAGEN DNeasy mini spin columns and reagents (QIAGEN, Valencia, CA), using the manufacturer's instructions for the isolation of total genomic DNA from gram-negative bacteria. DNA was quantified and tested for purity by measuring the absorbance spectrophotometrically at 260 nm and 280 nm. DNA from all isolations was dissolved and diluted in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The frequency of PCR gene targets was estimated to be one per genome copy. The number of copies of a *F. tularensis* genome per unit mass was calculated in the manner described below. An estimate of the genome size of *F. tularensis* (1.83 Mbp) was obtained from a recent analysis of the genome.[5]

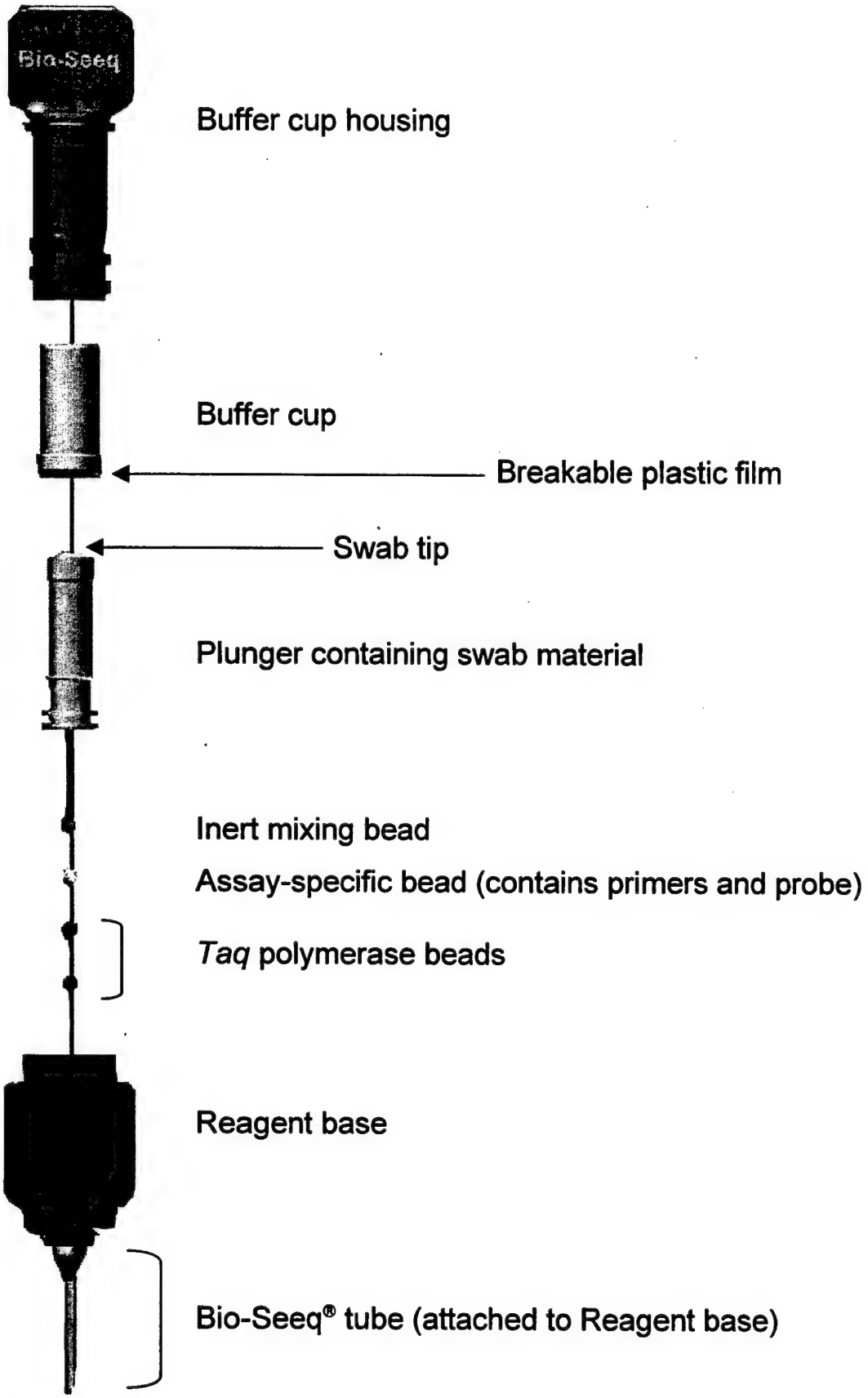

Figure 2. Consumable Sampling Device with Reaction Tube for the Bio-Seeq®

Table 1. Specificity of Reagents for *F. tularensis* Tested Against Genomic DNA

Source of target DNA[a]	No. of copies	No. positive/total[a]	Ct values[b]
Francisella tularensis LVS	1000	3/3	34, 35, 35
F. tularensis var. novicida	1000	6/6	31, 33, 33, 34, 35, 35
F. tularensis Schu 4	1000	3/3	32, 32, 33
F. tularensis 88R675	1000	3/3	37, 37, 38
F. tularensis A91-1623	1000	3/3	36, 37, 37
F. tularensis 88R144	1000	3/3	32, 32, 34
F. tularensis Salk	1000	3/3	30, 36, 36
Bacillus cereus	10^5	0/3	N/A
Bacillus subtilis	10^5	0/5	N/A
Bacteroides fragilis	10^5	0/3	N/A
Bordetella pertussis	10^5	0/3	N/A
Campylobacter jejuni	10^5	0/3	N/A
Clostridium perfringens	10^5	1/4	50
Clostridium tetani	10^5	0/3	N/A
Escherichia coli 43895	10^5	0/3	N/A
Escherichia coli 0157:H7	10^5	0/3	N/A
Neisseria meningitidis	10^5	0/3	N/A
Pseudomonas aeruginosa	10^5	0/3	N/A
Salmonella typhimurium LT2	10^5	0/3	N/A
Staphylococcus aureus 14458	10^5	0/3	N/A
Streptococcus pyogenes	10^5	0/3	N/A
Homo sapiens	10^5	0/3	N/A
synthetic target	1000	17/17	1@30, 1@35, 1@37, 6@38, 6@39, 1@41, 1@48
No template control	0	0/9	N/A

[a]Reactions run on either of two instruments. Data are combined from seven sets of experiments.
[b]N/A, not applicable. Values are reported only for positive assay results.

To calculate the mass of 1000 genomes:

1. Calculate Molecular Weight (MW):
   = 1.83 Mbp x 660 g/mole/bp = 1.21×10^9 g/mole of genomes

2. Calculate mass of 1000 genomes:

   1.21×10^9 g = 6.023×10^{23} genomes

   2.01×10^8 g = 10^{23} genomes (divide by 6.023)

   2.01×10^{-12} g = 10^3 genomes (divide by 10^{20})

Therefore, 1000 copies of a *Francisella tularensis* genome weigh approximately 2.0 pg.

4.3 Choice of Interferents.

Interferents were chosen by the marketing staff at SDE, in consultation with their marketing department. Four common materials were selected: coffee creamer (Domino Non-Dairy Creamer), baking powder (Rumford brand, Clabber Girl Inc., Terre Haute IN), wheat flour (America's Choice unbleached), and corn starch (Giant brand). Each material is found in most home kitchens and in many workplaces (especially coffee creamer). They may either be found on surfaces that have been contaminated with *F. tularensis* cells, or used by the perpetrator of a hoax, as it is widely believed that weaponized biological agents have the appearance of a white powder. All interferents were obtained by SDE staff at local supermarkets. Aliquots of each interferent were weighed and placed into 15 mL disposable plastic tubes before being supplied to the ECBC technical staff. Interferents were dissolved or suspended in nuclease-free, molecular biology grade water (Gibco BRL/Invitrogen, Carlsbad, CA) before use.

4.4 Assay Set-Up and Data Collection.

Assays were prepared in three ways:

a. To test the specificity and inclusivity of the probe and primer set using isolated total genomic DNA: for two reactions, a single dried bead containing the *F. tularensis* probe, primers, and internal control reagents was placed in a 1.5 mL microcentrifuge tube with two Ready-to-Go PCR reagent beads (containing *Taq* polymerase, dNTPs, and buffer) (Amersham Biosciences, Piscataway, NJ) and 48 μL nuclease-free water. When the reagent beads were dissolved, the mixture was divided into two 24 μL aliquots. One microliter of cells or DNA suspension was added to each reaction mixture (total volume 25 μL). Each reaction mixture was transferred to a Bio-Seeq® reaction tube, capped and covered with Parafilm, and gently centrifuged to draw the reaction mixture into the tube. Each tube was then placed into a separate PCR module in the Bio-Seeq® instrument and the thermocycling was started according to the manufacturer's instructions.

b. To test the limit of detection of the assay using viable cells as target, in an assembled consumable sampling device (a consumable; Figure 2): a single *F. tularensis* reagent bead and two Ready-to-Go PCR reagent beads were placed in a swab-tipped plunger with an inert mixing bead, and the plunger was inserted snuggly into the reagent base end of a consumable with a reaction tube affixed. Five microliters of a cell suspension (or DNA suspension or Tris buffer, in the control experiments) was spotted onto the surface of the swab tip. The consumable portion with the sample-bearing plunger, the reagents, and the reaction tube tip were then assembled with the portion containing the buffer cup. Twisting the two halves together broke the film on the buffer cup and allowed the sample and the reagent beads to be suspended in the buffer.

Assembled consumables were held inverted at room temperature for 90 s to allow the beads to dissolve, then shaken for 20 s to complete the dissolving and mix the reagents with the sample. The consumable was then "whipped" with a snapping motion of the wrist to drive a portion of the reaction mixture into the reaction tube. The reagent tube tip of the consumable was then inserted into one of the six PCR modules in the Bio-Seeq® instrument.

c. To test the effect of interferent materials on the ability of the assay to detect *F. tularensis* cells: reagent beads were inserted into a swab-tipped plunger, the plunger was assembled with the reagent base end of a consumable, and the swab was spotted with target suspension as described above. However, interferent powders in suspension were not easily or accurately delivered by pipette to the swab tip, making necessary the following variation. One hundred thirty microliters of an aqueous suspension of an interferent was added to empty unsealed buffer cups, which was then gently assembled with the swap-tipped plunger (to which a sample target had already been added). The buffer cup holder was then placed around the buffer cup and swab-tipped plunger. The entire consumable was then assembled, interferent-containing buffer dissolved the reagents in the plunger, and the rest of the assay was performed as described above.

5. RESULTS AND DISCUSSION

5.1 Specificity of Reagents for *Francisella tularensis*.

The assay for *F. tularensis* detects the presence of a sequence on the bacterial chromosome. Total genomic DNA was obtained from our in-house DNA collection or prepared from each species and strain tested as described above. To determine whether the assay accurately identified *F. tularensis* strains, we diluted preparations of genomic DNA from several strains to a concentration of 1000 genome copies per microliter. One microliter of each DNA suspension was added to 24 μL of reaction mixture as described above, placed in a reaction tube and inserted into the instrument. As positive controls, we also assembled reactions containing 1000 copies of synthetic target DNA designed to match the assay reagents. The PCR thermocycler profile was run as pre-programmed by SDE.

When prepared as described above, the assay detected 1000 copies of genomic DNA from several strains of *F. tularensis*, as well as 1000 copies of the synthetic target (Table 1). The specificity of the assay was also determined by testing large amounts of total genomic DNA (100,000 copies per assay) from several other species of bacteria, as well as human DNA (Table 1). No genomic DNA from the other species tested reacted positively in the *F. tularensis* assay.

One of four replicate tests of the *Clostridium perfringens* DNA produced a positive result on the Bio-Seeq®. The weakness of the result (positive result called on the last PCR cycle, giving a C_T of 50) and the lack of response in three other assays lead us to believe that this result was anomalous and that *C. perfringens* does not cross-react with the assay.

We also tested DNA from three other *F. tularensis* strains/varieties: *F. tularensis* v. philomoragia, *F. tularensis* strain 88R52, and *F. tularensis* type A. Each was detected in only 1 trial out of 3 per strain. Subsequent analysis of the target DNA by gel electrophoresis suggested, however, that these target DNAs may have become degraded during storage, and so these data are not reported in Table 1.

5.2 Limits of Detection Using Purified Genomic DNA from *F. tularensis* Strain LVS.

We determined the limit of detection for *F. tularensis* strain LVS DNA after noting that it reacted positively in the assay. *F. tularensis* strain LVS genomic DNA was diluted serially in TE buffer, and aliquots of each dilution were added to a reaction mixture as described above. As few as 62.5 copies of genomic DNA were consistently detected (Table 2). In the case of the single negative result when 50 copies of the genome were added to the assay, the trend in the data suggested that a positive result would have been reported in another few cycles (data not shown); if it had, the limit of detection in this study would have been reported as low as 30 copies per reaction. The cause of the single negative result in ten replicates of the synthetic target control is unknown.

5.3 Limits of Detection Using Cells of *F. tularensis* Strain LVS.

Laboratory assays for the detection of *F. tularensis* strain LVS cells, using the reagent bead set (without the consumable), were prepared as described in Materials and Methods. A suspension of cells was serially diluted in sterile 10 mM Tris HCl, pH 7.4 (Sigma Co., St. Louis, MO), and aliquots containing cells (or buffer alone) were added to PCR reaction mixtures prepared in microcentrifuge tubes, then placed in Bio-Seeq® reaction tubes. The reagent bead set, prepared as described, allowed the detection of as few as 1 cell per assay (Table 3). The cause of the two positive results in the negative control tubes is unknown, but conceivably may be due to operator error or contamination. It may also be notable that both results occurred in the same module of the same instrument (module three, instrument thermal paste), albeit on different days.

Table 2. Detection of *F. tularensis* Strain LVS Genomic DNA Using FT Reagent Beads[a]

No. of genome copies	No. positive/total[b]	Ct values[c]
1000	5/5	35, 35, 35, 35, 36
750	5/5	35, 35, 36, 36, 36
500	5/5	36, 37, 39, 41, 45
250	4/4	27, 38, 38, 38
125	6/6	39, 39, 40, 41, 41, 41
62.5	5/5	39, 39, 40, 40, 42
50	4/5	41, 41, 41, 41
40	5/5	40, 44, 46, 48, 48
30	5/5	38, 41, 42, 46, 48
20	3/8	45, 47, 49
15	6/8	43, 44, 46, 46, 48, 49
10	3/10	40, 45, 46
5	1/5	49
1	0/5	N/A
0 (buffer alone)	0/10	N/A
Synthetic target (1000 copies)	9/10	37, 4@38, 2@40, 41, 45

[a]Reaction mixtures prepared in a microcentrifuge tube and placed directly into a Bio-Seeq® reaction tube, without passing through the complete, assembled consumable sampling device. Results are combined from three experiments.
[b]Reactions run on either of two instruments.
[c]N/A, not applicable. Values are reported only for positive assay results.

Table 3. Detection of *F. tularensis* Strain LVS Cells Using FT Reagent Beads[a]

No. of colony-forming units	No. positive/total[b]	Ct values[c]
10^6	11/11	5@18, 4@19, 2@20
10^5	11/11	18, 20, 21, 3@22, 4@23, 25
10^4	11/11	2@25, 4@26, 5@27
5×10^3	11/11	7@27, 4@28
10^3	11/11	27, 4@30, 5@31, 32
10^2	11/11	32, 33, 2@34, 4@35, 2@36, 37
10^1	11/11	8@38, 3@39
1 cfu	11/11	2@41, 42, 2@44, 3@45, 2@47, 49
0 (buffer alone)	2/11	45, 46
P. agglomerans (10^5 cells)[d]	0/3	N/A

[a]Reaction mixtures prepared in a microcentrifuge tube and placed directly into a Bio-Seeq® reaction tube, without passing through the complete, assembled consumable sampling device. Results are combined from three experiments.
[b]Reactions run on either of two instruments.
[c]N/A, not applicable. Values are reported only for positive assay results.
[d]Species of gram-negative bacteria formerly known as *Erwinia herbicola*.

5.4 Limits of Detection and Specificity of the FT Assay Using the Consumable Sampling Device.

To examine the sensitivity of the assay for *F. tularensis* strain LVS cells when the reagent bead set is incorporated into the consumable sampling device, we performed the assays at several cell concentrations, applying cells to the consumable by spotting five-1 μL aliquots onto the swab tip. The data (Table 4) indicate that the assay for *F. tularensis* strain LVS cells has a LOD of approximately 10^3 cells per assay using the consumables. The cause of the negative results obtained when 1000 copies of the synthetic target were applied to the consumable is unknown; however, we did note in previous work with reagents for *Bacillus anthracis* that the assay for synthetic target was not as sensitive when using the consumable as when target was added to reaction mixtures prepared in microcentrifuge tubes. Perhaps similarly, the assay performed using the consumable is less sensitive than when it is assembled in microcentrifuge tubes. Approximately 10-fold fewer cells can be detected when assays are prepared in microcentrifuge tubes, relative to the number detectable when the consumable is used. We also applied 10^6 cfu of each of two nontarget organisms to the consumable; neither was detected by the assay (Table 4).

Table 4. Detection of *F. tularensis* Strain LVS Cells Applied to the Consumable Sampling Device

No. of colony-forming units	No. positive/total[a]	Ct values[b]
10^6	3/3	21, 23, 32
10^5	3/3	25, 30, 31
10^4	3/3	31, 34, 34
$5x10^3$	3/3	33, 34, 39
10^3	3/3	32, 36, 38
10^2	2/3	38, 41
10^1	2/3	42, 47
1 cfu	0/3	N/A
0 (no template control)	1/6	41
Escherichia coli O157:H7 (10^6 cfu)	0/3	N/A
Pantoea agglomerans (10^6 cfu)	0/3	N/A
synthetic target (1000 copies)[c]	1/5	32

[a]Reactions run on either of two instruments.
[b]N/A, not applicable. Values are reported only for positive assay results. Positive result in one NTC, no template control, experiment is unexplained; however, several false positive results were noted in the same module.
[c]1000 copies of synthetic target per reaction, applied to the complete, assembled consumable sampling device. Data are combined from two experiments.

5.5 Effects of Interferents in Laboratory Tests of the FT Assay.

Two concentrations of each inhibitor were used to test the effect of each on the ability of the assay to detect cells of *F. tularensis* strain LVS. The SDE staff determined the concentrations to be tested based upon work done at the SDE labs. The concentrations of cells to be tested were 10^3 cfu per assay (the limit of detection of the assay when the cells were applied to the consumable sampling device (Table 4), and 10-fold, 50-fold, and 100-fold excesses of that amount. All inhibitors were dissolved/suspended in nuclease-free water. All cell suspensions were diluted in 10 mM Tris HCl, pH 7.4. Tris diluent was used in place of cells in all of the negative controls.

Cornstarch had no effect on the performance of the assay when 5 mg were added (Table 5). When the amount of cornstarch was increased to 7.5 mg per assay, we observed that the limit of detection dropped somewhat (to between 10^3 and 5×10^3 cfu per assay).

Table 5. Detection of *F. tularensis* Strain LVS Cells Applied to the Consumable Sampling Device in the Presence of Cornstarch

Amount per assay[a]	No. of spores added	No. positive/total[b]	Ct values[c]
5 mg	10^4	3/3	34, 39, 41
	5×10^3	3/3	28, 34, 34
	10^3	3/3	39, 40, 42
	synthetic target (2000 copies)	1/1	21
	NTC	1/2	29
7.5 mg	10^4	3/3	21, 29, 38
	5×10^3	3/3	32, 35, 40
	10^3	1/3	37
	synthetic target (2000 copies)	1/1	20
	NTC	2/2	21, 22

[a]Total amount of interferent introduced into the consumable buffer cup in 130 µL of water. NTC, no-template control (negative control).
[b]Reactions run on either of two instruments.
[c]N/A, not applicable. Values are reported only for positive assay results.

Similarly, coffee creamer, at the lower concentration (10 mg per assay) had little effect on the performance of the assay (Table 6). However, the higher concentration of coffee creamer tested (15 mg per assay) did significantly degrade the ability of the assay to consistently detect cells of *F. tularensis*.

These results were consistent with the observations made while testing the effect of coffee creamer and cornstarch on the performance of the assay for *B. anthracis* spores,[6] in which 5 mg of cornstarch had no effect on assay performance.

Table 6. Detection of *F. tularensis* Strain LVS Cells Applied to the Consumable Sampling Device in the Presence of Coffee Creamer

Amount per assay[a]	No. of spores added	No. positive/total[b]	Ct values[c]
10 mg	10^4	3/3	29, 32, 40
	5×10^3	3/3	32, 40, 49
	10^3	3/3	28, 34, 44
	synthetic target (2000 copies)	0/1	N/A
	NTC	0/2	N/A
15 mg	10^4	2/3	29, 30
	5×10^3	1/3	33
	10^3	2/3	35, 37
	synthetic target (2000 copies)	0/1	N/A
	NTC	0/2	N/A

[a]Total amount of interferent introduced into the consumable buffer cup in 130 µL of water. NTC, no-template control (negative control).
[b]Reactions run on either of two instruments.
[c]N/A, not applicable. Values are reported only for positive assay results.

Likewise, 10 mg of coffee creamer did not significantly affect performance of the *B. anthracis* assay. As observed in the previous study,[6] an examination of the consumable suggests that the amounts of coffee creamer and cornstarch needed to partially inhibit the performance of the assay are far in excess of the amounts that would adhere to the swab tip when sampling a surface. We conclude from these results that neither coffee creamer nor cornstarch significantly inhibits the assay for *F. tularensis* in amounts likely to be introduced into the interior of the consumable.

Neither concentration of flour tested in this study (0.2 mg or 0.3 mg per assay) significantly affected the ability of the assay to detect cells of *F. tularensis* (Table 7). These results differ from those observed in the testing of the assay for *B. anthracis*, in which both concentrations inhibited some of the assays at each cell concentration tested. However, given the results observed in the study of the *B. anthracis* assay, we speculate that both flour and baking powder will inhibit the assay for *F. tularensis* in quantities far less than coffee creamer or cornstarch. This inhibition is probably due, at least in part, to the presence of high molecular weight polysaccharides (mostly starches, in white flour) and protein (gluten). Both of these components contribute to the tendency of flour to form a sticky suspension at higher concentrations (eventually approaching the consistency of dough).

Table 7. Detection of *F. tularensis* Strain LVS Cells Applied to the Consumable Sampling Device in the Presence of Wheat Flour

Amount per assay[a]	No. of spores added	No. positive/total[b]	Ct values[c]
0.2 mg	10^4	3/3	32, 34, 40
	5×10^3	3/3	28, 36, 37
	10^3	3/3	22, 35, 42
	synthetic target (1000 copies)	0/1	N/A
	NTC	0/2	N/A
0.3 mg	10^4	3/3	35, 35, 41
	5×10^3	3/3	33, 35, 39
	10^3	3/3	36, 37, 44
	synthetic target (2000 copies)	1/1	43
	NTC	0/2	N/A

[a]Total amount of interferent introduced into the consumable buffer cup in 130 µL of water. NTC, no-template control (negative control).
[b]Reactions run on either of two instruments.
[c]N/A, not applicable. Values are reported only for positive assay results.

Even smaller amounts of baking powder inhibited the performance of the *F. tularensis* assay (Table 8). The minimum inhibitory concentration of baking powder observed in the study of the *B. anthracis* assay was between 0.025 and 0.0025 mg per assay when 10^4 spores were present in the assay. In this study we tested only the two highest concentrations, 0.050 and 0.075 mg baking powder per assay. Even at the highest cell concentrations we observed significant inhibition of the *F. tularensis* assay. As before, we hypothesize that the inhibition by baking powder is the result of some combination of changes in the pH of the reaction solution brought about by the acid-base reaction of baking powder components with water and generation of CO_2 gas by the same reaction. Gas bubbles in Bio-Seeq® tubes are perhaps the single greatest cause of experimental artifacts (false positive and false negative results). Another component of some baking powders (including that used in this work), calcium acid phosphate ($Ca(H_2PO_4)_2$) contains pyrophosphate, which may also inhibit *Taq* polymerase because it is a product of the DNA polymerization reaction. Some flours (self-rising flour) contain baking powder; the effect of these two interferents combined in this fashion was not examined in this study.

Table 8. Detection of *F. tularensis* Strain LVS Cells Applied to the Consumable Sampling Device in the Presence of Baking Powder

Amount per assay[a]	No. of spores added	No. positive/total[b]	Ct values[c]
0.05 mg	10^4	1/3	33
	5×10^3	0/3	N/A
	10^3	0/3	N/A
	synthetic target (2000 copies)	1/1	47
	NTC	1/2	26
0.075 mg	10^4	0/3	N/A
	5×10^3	3/3	20, 46, 47
	10^3	1/3	34
	synthetic target (2000 copies)	1/1	38
	NTC	0/2	N/A

[a]Total amount of interferent introduced into the consumable buffer cup in 130 μL of water. NTC, no-template control (negative control).
[b]Reactions run on either of two instruments.
[c]N/A, not applicable. Values are reported only for positive assay results.

6. CONCLUSIONS

The Bio-Seeq® instrument and reagents for *F. tularensis* assays correctly identified *F. tularensis* strains containing the target DNA sequence, and did not react with 15 other species and strains of bacteria, nor with human DNA. Under laboratory conditions, the sensitivity of the assay was observed to be as low as 30-62 copies of the genome (comparable to the LOD of 100 copies reported by Emanuel et al.).[3] The LOD for intact cells was determined in this study to be as low as 1 cell per assay (without consumable; the observed LOD while using the consumable was 1000 cells).

The addition of coffee creamer or cornstarch had little effect on the performance of the assay under the conditions tested; however, baking powder adversely affected assay performance, reducing the sensitivity several orders of magnitude when the assay was performed using the consumable sampling device. The presence of flour was not observed to inhibit the performance of the *F. tularensis* assay in this study; however, we anticipate that concentrations of flour slightly higher than those used here would inhibit the assay, given results observed while testing the *B. anthracis* assay.

It must be pointed out that no sample preparation or DNA clean-up steps were involved in the assays performed in this study, although the heat of thermocycling is likely to degrade the integrity of cells of gram-negative bacteria. Curiously, a comparison of the LOD for genome copies (purified DNA) versus the results for cells shows that the LOD for purified DNA was some 30-100 times higher than the LOD for cells. This counterintuitive result might be explained in either of two ways. It is possible

that isolated genomic DNA was partially degraded, resulting in an overestimation of the number of targets per mass of DNA. Alternatively, it is possible that DNA containing target sequences was clinging to the outside of cells, or carried along in the cell preparation from the initial isolation (since no steps were taken to break open cells to release the DNA contained inside). Further sample processing (resulting in recovery of most of the sample DNA in pure form) should increase the accuracy of the assay, as well as remove many of the problems caused by the presence of interferents.

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